*BACTERIOPHAGE T4 DNA-DEPENDENT IN VITRO SYNTHESIS OF LYSOZYME**

By Manfred Schweiger and Lawrence M. Gold

THE ROCKEFELLER UNIVERSITY

Communicated by Fritz Lipmann, June 9, 1969

Abstract.—A cell-free system derived from uninfected *Escherichia coli* previously was shown to synthesize β-glucosyl transferase in response to T4 DNA. This same *in vitro* system, when incubated at slightly higher magnesium concentrations, also synthesized enzymatically active lysozyme. The lysozyme activity that appeared was judged to be T4-specific since antibodies prepared against authentic T4-lysozyme inactivated the *in vitro* synthesized enzyme. DNA from a T4 mutant carrying a deletion in the lysozyme gene stimulated amino acid incorporation to the same extent as wild-type T4 DNA but was inactive in directing the synthesis of lysozyme. Various inhibitors of RNA and protein synthesis inhibited the *in vitro* synthesis of lysozyme.

Upon infection of a sensitive strain of *Escherichia coli* by the bacteriophage T4, there occurs a rigidly controlled sequence of events that leads to the development and release of mature virus particles.[1-3] Depending on the kinetics of their appearance, most of the proteins synthesized after T4 infection may be classified as either early or late. The early enzymes, such as β-glucosyl transferase, appear one to two minutes after infection, whereas the late proteins, such as lysozyme, do not appear until eight minutes after infection.[4, 5]

By the use of components isolated from uninfected *E. coli*, we previously demonstrated the *in vitro* DNA-dependent synthesis of β-glucosyl transferase, an early enzyme.[6] Since mRNA isolated from T4-infected cells had been shown to stimulate lysozyme synthesis *in vitro*,[7] we looked for T4 DNA-dependent lysozyme synthesis in cell-free systems known to be active for β-glucosyl transferase synthesis. *De novo* synthesis of lysozyme did occur in response to T4 DNA, thereby demonstrating that a region of the DNA corresponding to a late protein can be transcribed and translated by the enzymes of uninfected *E. coli*.

Methods.—Cell-free system: All methods for the preparation of bacteria and bacteriophages, as well as the preparation of ribosomes, supernatant enzymes, and phage DNA were described previously.[6] DNA-dependent RNA and protein synthesis reactions were incubated as described for the cell-free synthesis of β-glucosyl transferase,[6] except that the magnesium ion concentration was raised to 15 mM. *E. coli* K12AB301 or 514 was used to prepare the cell-free extracts.

Uninfected and T4-infected E. coli extracts: *E. coli* K110 was grown to 5×10^8 cells/ml. Infection by T4 was at a multiplicity of infection of 3. After 25 min at 30°, infected cells and uninfected cells were cooled with ice, harvested, and washed once. The cells were resuspended in 10 mM Tris-HCl, pH 7.8 + 10 mM magnesium acetate + 50 mM ammonium chloride + 1 mM dithiothreitol, sonicated for 30 sec with a Branson sonifier, and centrifuged at 30,000 × *g* for 20 min. The supernatant fluids were stored frozen until needed.

Substrate for radiochemical lysozyme assay: We are grateful to Drs. W. Leutgeb and U. Schwarz for sending instructions for the lysozyme assay prior to publication. *E. coli* were grown to stationary phase in 10 ml of Fraser and Jerrel[8] medium in the presence of

100 μc of tritiated 1,6-diaminopimelic acid. Aliquots of 0.1 ml were spotted on 2.5-cm discs of Whatman 3 MM filter paper, which were washed according to the method of Mans and Novelli.[9] The discs were then boiled for 10 min in 0.1 *M* ammonium acetate, followed by a final wash in fresh ammonium acetate. The filters were stored in the cold until needed. Routinely, 70,000 to 100,000 cpm of radioactive material remain fixed to the filters. As seen in Table 3, about half the radioactivity can be released by egg-white lysozyme and the remainder by trypsin. This is consistent with the fate of diaminopimelic acid taken up by *E. coli*, since Meadow and Work[10] have demonstrated that a large portion of that diaminopimelic acid is converted to lysine and presumably incorporated into protein.

Lysozyme assay: Reaction mixtures from *in vitro* protein synthesis were incubated with radioactive filter discs plus 0.5 ml of 0.1 *M* ammonium acetate. After 4 hr at 37°, aliquots of 0.3 ml were removed, added to 10 ml of Bray's solution,[11] and counted in a liquid scintillation counter. When aliquots corresponding to 500 μg of ribosomes were assayed, reaction mixtures that had been incubated in the presence of T4 DNA released 8,000 to 20,000 cpm from the filters. After the background due to *E. coli* proteins in the *in vitro* components was subtracted, lysozyme synthesis was expressed as cpm released/hr/250 μg ribosomes. The background in these experiments was about 1500 cpm/0.3-ml aliquot. The background was subtracted from all assays before the amount of lysozyme synthesized was calculated. Many of the experiments reported in this paper have been repeated with the standard spectrophotometric assay for lysozyme.[7] No qualitative differences were observed in the comparison. However, the radioactive substrate is far more stable than chloroform-sensitized cells, so that the reproducibility of the radiochemical assay is much higher than that of the turbidity assay. Since the sensitivity of the radiochemical assay is sufficient, we now use it routinely in this work.

Materials.—T4eG59 and *E. coli* K110 were given to us by Dr. George Streisinger. Antibodies against T4 lysozyme were prepared and donated by Dr. Yoshimi Okada.

Results.—When the components of the *in vitro* system were incubated in the presence of T4 DNA, amino acid incorporation into protein was stimulated about sixfold. Whereas the components of the cell-free system had no detectable lysozyme activity before incubation at 37°, the complete mixtures contained lysozyme activity after 30 minutes of *in vitro* RNA and protein synthesis. Lysozyme activity did not appear if the cell-free system was stimulated by DNA from the bacteriophage T4eG59, a mutant containing a deletion in the lysozyme gene (Table 1). DNA from the mutant bacteriophage was fully active in the stimulation of amino acid incorporation and was also active in the *in vitro*

TABLE 1. *Lysozyme synthesis in vitro in response to T4 DNA.*

	In vitro system Additions for protein synthesis	Amino acid incorporation (pmole C^{14}-leu/250 μg ribosome)	Lysozyme activity (cpm/hr/250 μg ribosomes)
I	None	135	<50
	T4 DNA	920	1060
	T4 DNA; without 37° incubation	18	<50
	T4eG59 DNA	940	<50
II	None	105	<50
	T4 DNA	630	2300
	T4eG59 DNA	590	<50

Protein synthesis was carried out as described previously.[6] Incubations were for 30 min at 37°, after which the lysozyme activities were measured. Parallel protein synthesis reactions were incubated in the presence of C^{14}-leucine (25 μc/μmole) so that amino acid incorporation could be measured. The cell-free systems I and II were prepared from *E. coli* K12AB301 and 514, respectively. DNA concentrations were 30 μg/ml.

synthesis of β-glucosyl transferase (not shown). Thus, the absence of lysozyme synthesis using DNA from T4G59 was due to its genetic lesion rather than to spurious damage incurred during bacteriophage preparation and deproteinization. The data presented in Table 1 show that the ratio of enzyme synthesis to amino acid incorporation varied from one cell-free system to the next. This phenomenon was observed previously in the *in vitro* synthesis of β-glucosyl transferase.[6] Although different strains of *E. coli* were used to prepare the two extracts used in Table 1, the differences are not due to peculiarities in the strains but rather to variability in the preparation of ribosomes and supernatant proteins.

Although experiments with DNA from T4eG59 provided a suitable and useful control, lysozyme was further identified as one product of T4 DNA-dependent protein synthesis in two other ways. The lysozyme activity resulting from *in vitro* protein synthesis was shown to be completely inactivated by antiserum against authentic homogeneous T4 lysozyme. The lysozyme activity found in T4-infected cells was similarly inactivated by the antibodies, whereas equivalent amounts of egg-white lysozyme were not affected (Table 2). We have also assayed lysates prepared from cells infected with T4eG59, and, as expected, very little radioactivity was released from the filters. Uninfected extracts were also unable to release large amounts of radioactivity. Thus, under these conditions, the filter assay appeared to be specific for lysozyme, and the lysozyme activity which arose *de novo* in the DNA-dependent reaction was inactivated by antibodies prepared against T4-specific lysozyme.

In a second type of control experiment, we asked whether the lysozyme activity that resulted from DNA-dependent RNA and protein synthesis would compete with authentic T4-specific lysozyme or egg-white lysozyme for the radioactive substrate immobilized on the paper filters. Filters containing 106,000 cpm of

TABLE 2. *Inactivation (by specific antibodies) of T4 lysozyme synthesized in vivo and in vitro.*

Enzyme	Antibody pretreatment (μg)	Lysozyme activity (cpm)
Extract from uninfected *E. coli*	—	1,420
Extract from T4eG59-infected *E. coli*	—	1,560
Extract from T4-infected *E. coli*	—	16,783
Extract from T4-infected *E. coli*	50	1,337
Extract from T4-infected *E. coli*	100	1,865
T4 DNA-directed *in vitro* proteins	—	11,482
T4 DNA-directed *in vitro* proteins	50	2,714
T4 DNA-directed *in vitro* proteins	100	2,500
T4eG59 DNA-directed *in vitro* proteins	—	1,872
Egg-white lysozyme	—	15,000
Egg-white lysozyme	50	20,000
Egg-white lysozyme	100	18,800
50 μg anti-T4-lysozyme serum	—	1,014
100 μg anti-T4-lysozyme serum	—	2,184

The amount of crude extract assayed corresponded in each case to 6×10^7 cells. Aliquots of the *in vitro* reactions corresponding to 500 μg of ribosomes were used, whereas 50 ng of egg-white lysozyme were used. Pretreatment with antibodies consisted of a 5-min incubation at 37°. Lysozyme assays were incubated for 4 hr (see *Methods*), after which 0.3-ml aliquots were counted.

washed substrate (see *Methods*) were exhaustively treated with either egg-white lysozyme, trypsin, uninfected *E. coli* extracts, T4-infected *E. coli* extracts, or *in vitro* reaction mixtures incubated with T4 or T4eG59 DNA. The *in vitro* reaction mixtures had been incubated for 30 min at 37° to allow lysozyme synthesis to occur. Twelve-hour incubations at 37° were used for the exhaustive pretreatment of the filters, after which they were washed and boiled in 0.1 *M* ammonium acetate. These pretreated filters were then incubated in a second exhaustive reaction with each of the various protein fractions. The amount of radioactivity released during the second exhaustive treatment was measured (Table 3). The

TABLE 3. *Substrate specificity of T4 lysozyme synthesized in vitro.*

	Second Treatment					
Pretreatment	Egg-white lysozyme	Trypsin	T4-infected *E. coli* extract	Uninfected *E. coli* extract	T4 DNA-directed *in vitro* proteins	T4eG59 DNA-directed *in vitro* proteins
None	52,000	47,700	54,300	3,000	40,900	4,900
Egg-white lysozyme	480	48,000	1,470	1,200	2,100	2,100
Trypsin	60,500	2,500	54,000	3,500	51,000	6,600
T4-infected *E. coli* extract	1,050	53,500	2,400	1,350	2,300	2,200
Uninfected *E. coli* extract	52,500	41,000	—	—	40,000	3,600
T4 DNA-directed *in vitro* proteins	7,900	62,000	11,400	2,000	7,500	4,000
T4eG59 DNA-directed *in vitro* proteins	51,500	44,000	—	—	35,200	3,500

Substrate filters for the lysozyme assay, each containing 106,000 cpm, were incubated for 12 hr at 37° with 0.5 ml of 0.1 *M* ammonium acetate, plus either 5 μg of egg-white lysozyme, 10 μg of trypsin, extracts from T4-infected or uninfected *E. coli* corresponding to 3×10^8 cells, or *in vitro* reaction mixtures containing 500 μg of ribosomes which had been stimulated by either T4 or T4eG59 DNA. After the pretreatment, the total amount of radioactivity released was determined (*top row*). Pretreated filters were then washed and boiled in 0.1 *M* ammonium acetate, after which they were incubated a second time with each of the protein fractions in 0.5 ml ammonium acetate. After 12 hr at 37°, the total amount of radioactivity released was again determined (*bottom six rows*). The data are given as cpm released/0.5 ml reaction.

least complicated interpretation of this experiment suggested that egg-white lysozyme and T4-specific lysozyme attacked the same radioactive material on the filter substrates and that the lysozyme synthesized *in vitro* behaved in the same manner as the enzyme present in extracts of T4-infected cells. Furthermore, about one half of the radioactivity on the filters was released by trypsin, presumably representing peptides with radioactive lysine.[10] The trypsin-sensitive material is not attacked by either egg-white lysozyme or T4 lysozyme synthesized *in vivo* or *in vitro*. The evidence obtained with the lysozyme deletion mutant, anti-T4-lysozyme serum, and exhaustive treatment of the filter substrates suggested that T4-specific lysozyme was synthesized *in vitro*.

Various inhibitors of RNA and protein synthesis were tested to determine whether lysozyme appearance *in vitro* was always dependent on transcription and translation (Table 4). Each inhibitor eliminated the T4 DNA-dependent stimulation of amino acid incorporation and, at the same time, abolished lysozyme synthesis. None of the inhibitors inhibited the activity of T4 lysozyme.

TABLE 4. *Effect of inhibitors of RNA and protein synthesis on lysozyme appearance.*

Additions for protein synthesis	Amino acid incorporation (pmole/250 μg ribosomes)	Lysozyme activity (cpm/hr/250 μg ribosomes)
None	68	<50
T4 DNA	402	1095
T4 DNA + actinomycin D (17 μg/ml)	64	<50
T4 DNA + puromycin (10^{-3} *M*)	7	<50
T4 DNA + chloramphenicol (50 μg/ml)	59	<50
T4 DNA + DNase (10 μg/ml)	72	<50

Protein synthesis was carried out for 30 min at 37°. T4 DNA was added, when required, at 30 μg/ml. All inhibitors were added to the reaction mixtures just before the start of the incubations.

All the DNA-dependent *in vitro* reactions described thus far were carried out at DNA concentrations of 30 μg/ml or higher. This was sufficient to saturate the *in vitro* system for lysozyme synthesis (Fig. 1). Lysozyme synthesis was proportional to T4 DNA concentrations up to 15 μg/ml. This was lower than the saturating level of DNA reported previously for β-glucosyl transferase synthesis.[6] In fact, we find the saturating level of DNA for enzyme synthesis to be dependent on the particular set of ribosomes and S-100 protein fraction, so that no inferences may be made concerning differential levels of DNA saturation for early versus late enzyme synthesis.

The kinetics of lysozyme appearance during T4 DNA-dependent protein synthesis (Fig. 2) were qualitatively similar to the kinetics of β-glucosyl transferase synthesis. In each case, enzyme activity appeared after a short lag period, whereas amino acid incorporation proceeds maximally from time zero. The rapid degradation of β-glucosyl transferase activity after amino acid incorporation had stopped was not seen in the case of lysozyme.[6]

The *in vitro* reactions for lysozyme synthesis were done at 15 mM magnesium acetate, whereas for β-glucosyl transferase synthesis, 11 mM magnesium acetate was used.[6] The magnesium profiles for maximal rates of synthesis of the two enzymes are shown in Figure 3. Whereas β-glucosyl transferase synthesis was sharply inhibited above 12 mM Mg^{++}, lysozyme synthesis was highest in the range of 14–16 mM Mg^{++}. These curves did not represent the effect of magnesium acetate on enzymatic activity in the subsequent assay, as our unpub-

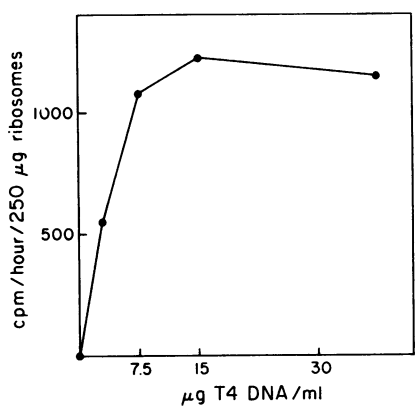

FIG. 1.—T4 DNA saturation for lysozyme synthesis *in vitro*. Protein synthesis reaction mixtures were incubated with increasing amounts of T4 DNA. After 30 min at 37°, aliquots were assayed for lysozyme activity (see *Methods*).

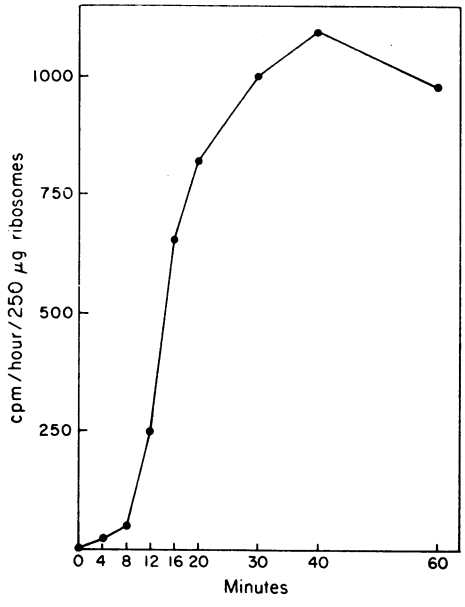

FIG. 2.—Kinetics of lysozyme synthesis *in vitro*. A large-scale reaction mixture containing T4 DNA at 30 μg/ml was incubated for protein synthesis at 37°. At various times, aliquots were removed and assayed for lysozyme activity.

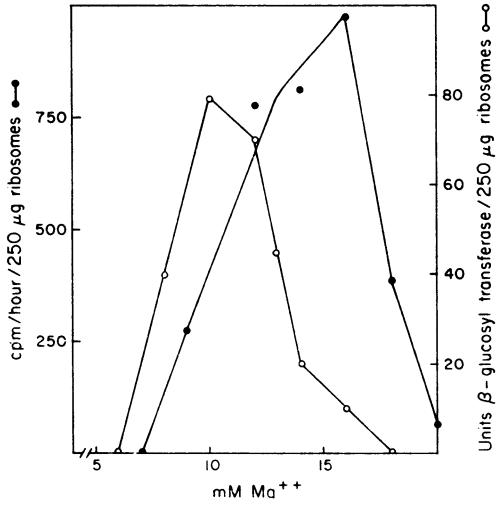

FIG. 3.—Magnesium ion requirements for lysozyme and β-glucosyl transferase synthesis. Protein synthesis reaction mixtures containing T4 DNA were incubated with increasing Mg^{++} concentrations. After 30 min at 37°, aliquots were removed and assayed for β-glucosyl transferase as well as lysozyme.

lished experiments showed that each of the enzymes, when present in extracts of T4-infected *E. coli*, was relatively insensitive to changes in magnesium ion concentration in this range. When the magnesium profile for amino acid incorporation was measured in the presence of T4 DNA, the curve obtained was qualitatively similar to that for lysozyme synthesis rather than β-glucosyl transferase synthesis.[12]

Discussion.—The evidence presented in this paper suggests that extracts prepared from uninfected *E. coli* can transcribe and translate the lysozyme gene of T4 DNA. The *in vitro* synthesis of active lysozyme has the characteristics required of *de novo* enzyme synthesis. Inhibitors of RNA and protein synthesis

abolished lysozyme synthesis. Furthermore, routine platings of the cell-free system revealed no viable cells, and the DNA preparations had no viable bacteriophages.

The enzyme activity that resulted from *in vitro* synthesis was identical to T4 lysozyme by several criteria. The DNA from T4eG59, a mutant carrying a deletion in the lysozyme gene, was inactive in the synthesis of lysozyme although it was completely active for the stimulation of amino acid incorporation (Table 1) and β-glucosyl transferase synthesis. Antibodies prepared against purified T4 lysozyme inactivated the enzyme synthesized *in vitro* and *in vivo*, while they barely affected the activity of equivalent amounts of egg-white lysozyme (Table 2). This was not surprising in view of the many physical differences between egg-white and T4-specific lysozymes.[13] Finally, egg-white lysozyme, T4 lysozyme synthesized *in vivo*, and T4 lysozyme synthesized *in vitro* each released the same materials from the substrate filters used in the radiochemical lysozyme assay (Table 3).

Lysozyme has been reported to be a late T4-specific enzyme, since only after eight to ten minutes can any enzyme activity be detected in T4-infected cells.[5] If, in fact, lysozyme was a typical late protein, one might expect our *in vitro* system not to make detectable amounts of it, as several laboratories have shown that the *in vitro* reaction between T4 DNA and *E. coli* RNA polymerase results in the synthesis of only early mRNA.[14–17] Salser and co-workers have shown that early mRNA isolated from infected cells does not stimulate lysozyme synthesis *in vitro*, whereas late mRNA prepared in the same manner did stimulate lysozyme synthesis.[7]

Lysozyme, however, is clearly not a typical late protein. Streisinger's group[18] has shown that the direction of transcription and translation of the lysozyme gene is the same as that of the early gene rII. Furthermore, the lysozyme-specific mRNA is synthesized on the same strand of DNA as the bulk of the early messenger.[19] Recent evidence from Bautz's laboratory suggests that lysozyme-specific mRNA is synthesized at early times in amounts comparable to those found late in infection, although the early lysozyme-specific messenger is not translated.[20] Thus, the expression of the lysozyme gene is subject to controls which are not necessarily similar to the control of most early and late functions.

The kinetics and T4 DNA saturation for lysozyme synthesis *in vitro* was found to be qualitatively similar to β-glucosyl transferase (Figs. 1 and 2; ref. 6). In fact, no obvious differences have been observed in the requirements for the synthesis of the two enzymes, with the exception of optimal Mg^{++} concentration (Fig. 3). The reason for the difference in optimum Mg^{++} concentration is entirely unknown at this time.

The *in vitro* system synthesizes equivalent amounts of β-glucosyl transferase and lysozyme. By conducting *in vitro* protein synthesis at the Mg^{++} optimums for each enzyme, we have calculated that 0.050-ml reaction mixtures synthesize as much β-glucosyl transferase and lysozyme as is found in 0.3 to 1.1 $\times 10^7$ cells infected for 15 min and 1 to 3 $\times 10^7$ cells infected for 25 min, respectively. This suggests that the components from uninfected cells do not discriminate against

the transcription and translation of the lysozyme gene, as long as suitable Mg^{++} concentrations are used. The efficiency of the *in vitro* system is low when compared with infected cells. Whereas *de novo* enzyme synthesis occurs in amounts equivalent to $\sim 10^7$ infected cells, the amount of ribosomes used is equivalent to more than 10^9 infected cells.

We are most grateful to Dr. Fritz Lipmann for giving us the opportunity to work in his laboratory and to discuss these experiments with him. We also thank Dr. W. Leutgeb for making available to us his assay procedure for lysozyme, and Drs. G. Streisinger and J. Gordon for many helpful discussions.

* This work was supported, in part, by a grant from the USPHS (GM-13972) and by fellowships from the USPHS (5 FO2 GM36140) to L. M. G. and from the Deutscher Akademischer Austauschdienst to M. S.

[1] Levinthal, C., J. Hosoda, and D. Shub, in *Molecular Biology of Viruses*, ed. J. S. Colter and W. Paranchych (New York: Academic Press, 1967), p. 71.

[2] McCorquodale, D. J., A. E. Oleson, and J. M. Buchanan, in *Molecular Biology of Viruses*, ed. J. S. Colter and W. Paranchych (New York: Academic Press, 1967) p. 31.

[3] Campbell, A., in *Molecular Genetics*, ed. J. H. Taylor (New York: Academic Press, 1967), vol. 2, p. 323.

[4] Kornberg, S. R., S. B. Zimmerman, and A. Kornberg, *J. Biol. Chem.*, **236**, 1487 (1961).

[5] Protass, J. J., and D. Korn, these PROCEEDINGS, **55**, 832 (1966).

[6] Gold, L. M., and M. Schweiger, these PROCEEDINGS, **62**, 892 (1969).

[7] Salser, W., R. F. Gesteland, and A. Bolle, *Nature*, **215**, 588 (1967).

[8] Fraser, D., and E. A. Jerrel, *J. Biol. Chem.*, **205**, 291 (1953).

[9] Mans, R. J., and G. D. Novelli, *Arch. Biochem. Biophys.*, **94**, 48 (1961).

[10] Meadow, P., and E. Work, *Biochem. J.*, **72**, 400 (1959).

[11] Bray, G., *Anal. Biochem.*, **1**, 249 (1960).

[12] Gold, L. M., and M. Schweiger, *J. Biol. Chem.*, in press.

[13] Tsugita, A., M. Inouye, E. Terzaghi, and G. Streisinger, *J. Biol. Chem.*, **243**, 391 (1968).

[14] Hall, B. D., A. P. Nygaard, and M. H. Green, *J. Mol. Biol.*, **9**, 143 (1964).

[15] Geiduschek, E. P., L. Snyder, A. J. E. Colvill, and M. Sarnat, *J. Mol. Biol.*, **19**, 541 (1966).

[16] Khesin, R. B., Zh. M. Gorlenkow, M. F. Shemyakin, I. A. Bass, and A. A. Prosorow, *Biokhimiya*, **28**, 1010 (1963).

[17] Traub, P., W. Zillig, R. L. Millette, and M. Schweiger, *Z. Physiol. Chem.*, **343**, 261 (1966).

[18] Streisinger, G., J. Emrich, Y. Okada, A. Tsugita, and M. Inouye, *J. Mol. Biol.*, **31**, 607 (1968).

[19] Kasai, T., E. K. F. Bautz, A. Guha, and W. Szybalski, *J. Mol. Biol.*, **34**, 709 (1968).

[20] Kasai, T., and E. K. F. Bautz, *J. Mol. Biol.*, **41**, 401 (1969).